

ATOMIC FORCE MICROSCOPY AS AN ADVANCED TOOL IN NEUROSCIENCE

Abstract

This review highlights relevant issues about applications and improvements of atomic force microscopy (AFM) toward a better understanding of neurodegenerative changes at the molecular level with the hope of contributing to the development of effective therapeutic strategies for neurodegenerative illnesses. The basic principles of AFM are briefly discussed in terms of evaluation of experimental data, including the newest PeakForce Quantitative Nanomechanical Mapping (QNM) and the evaluation of Young's modulus as the crucial elasticity parameter. AFM topography, revealed in imaging mode, can be used to monitor changes in live neurons over time, representing a valuable tool for high-resolution detection and monitoring of neuronal morphology. The mechanical properties of living cells can be quantified by force spectroscopy as well as by new AFM. A variety of applications are described, and their relevance for specific research areas discussed. In addition, imaging as well as non-imaging modes can provide specific information, not only about the structural and mechanical properties of neuronal membranes, but also on the cytoplasm, cell nucleus, and particularly cytoskeletal components. Moreover, new AFM is able to provide detailed insight into physical structure and biochemical interactions in both physiological and pathophysiological conditions.

Keywords

• Atomic force microscopy • Force spectroscopy • Membrane nanomechanics
• Neuron • Neuroscience • PeakForce Quantitative Nanomechanical Mapping

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Introduction

The human brain is organized as a complex network of neuronal circuits and brain areas that process information based on specific patterns of spatial and temporal neuronal activity [1, 2]. Functional abilities of the brain such as perception and integration of sensory information from diverse modalities, control of body movement and higher processes including cognition, attention, language comprehension, decision making, long-term planning, and problem solving are determined by the functional and structural properties of interconnected neurons.

Neurons, the electrically excitable cells of the brain, are specialized for fast processing and transmission of multiple types of information to and from the brain, and have specialized structures for intercellular communication [3-5]. Neurodegenerative diseases, like Alzheimer's disease and Parkinson's disease, are characterized by compromised function

of the central nervous system due to progressive neuronal damage and loss. Damaged neurons are unable to communicate efficiently, which ultimately results in diverse clinical phenotypes characteristic for each particular neurodegenerative disease [6]. Despite major research efforts, there is still no effective disease-modifying therapy for neurodegenerative diseases.

Neuronal loss in vulnerable, disease-specific brain areas is a relatively late event in the progression of neurodegenerative disorders. It is usually preceded by subtle structural and associated functional changes, including synaptic loss, neurite retraction, and disturbance of axonal transport due to microtubule rearrangement and instability [7-10]. The latter occurs in conjunction with the appearance of slight changes at the molecular level, such as peroxidation of membrane lipids, DNA oxidation and protein aggregation; the last one being the most prominent and researched pathological

hallmark of the neurodegenerative diseases [11-14].

For the most commonly used and well-established research techniques in neuronal studies, it is challenging to determine the key structural changes at the molecular level that fundamentally contribute to the onset and progression of neurodegenerative diseases or regeneration after neuronal injury [15-17]. The structural and nanomechanical properties of neurons are affected by cytoskeletal components (microtubules, neurofilaments, and actin filaments), the cytoplasm, the cell nucleus, and by the neuron-extracellular matrix interactions that are important for neuron-substrate adhesion processes, axonal growth and neuronal migration [18]. Therefore, there is a growing interest in the application and improvement of novel and advanced techniques in the research of neurodegenerative changes, and neuronal injury and repair, at the molecular level and at the nanoscale, in the context of the development of novel therapeutic strategies.

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The atomic force microscope is at the forefront of such studies. In general, atomic force microscopy (AFM) covers a large research area, including plant and animal cells, bacteria and viruses, as well as proteins and other molecules (Fig. 1). This paper aims to cover recent progress in the study of neuronal cells and their subcellular structures using modern AFM techniques. We review the potential of the AFM techniques with the simultaneous evaluation of the local nanomechanical properties and the neuronal cell topography at a high spatial resolution and force sensitivity, as well as in understanding the development of the nervous system [19]. It should be pointed out that the field of AFM application in neuronal investigation is developing, and the present review is neither comprehensive nor final, as it covers only a selection of recent investigations.

Basic principles of AFM

The atomic force microscope was invented in 1986 by Binnig, Quate and Gerber. Similar to other scanning probe microscopes, the atomic force microscope raster-scans a sharp probe over the surface of a sample and measures the changes in atomic forces between the probe tip and the sample. Figure 2 illustrates the working concept of the atomic force microscope. The interaction between the sample surface and the probe tip corresponds to the forces between the atoms of the sample and the tip that scans the sample's surface. In the contact area of the tip apex, repulsion occurs due to the overlapping electronic shells of the tip and sampled atoms

[20, 21]. With an AFM tip, it is possible to probe very small interaction areas (the radius of the tip is in the range of 5-50 nm), which ensures high sensitivity to small forces. To make these forces accessible, the tip is placed on a very soft spring, the AFM cantilever. Depending on the separation distance between the probe and sample, long- or short-range forces will dominate the interaction. These forces are measured by the bending or twisting of the cantilever by a laser beam, which is focused on the back of a cantilever, and then reflected into a photodiode detector. Any forces acting on the AFM tip cause deflection of the cantilever. Small forces between the tip and sample will cause less deflection than large forces, and forces in the pN range can be measured. These correspond to the order of magnitude of forces that are required to separate ligand from receptor. Finally, deflection of the cantilever after interaction with the sample surface is translated into a three-dimensional (3D) image of the surface.

AFM measures surface forces such as mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces, and suchlike [22-25]. AFM probes are composed of flexible, triangular or rectangular cantilevers with a sharp tip near the end of the cantilever (Fig. 3). They can be manufactured from a variety of materials, but most AFM probes are made from silicon and/or silicon nitride (Si_3N_4) wafers. An AFM probe's sensitivity, or spring constant (k), is the force required to bend the cantilever per unit distance (usually expressed in N/m). It is an important parameter in AFM probe behaviour

and performance for imaging applications, and an essential factor when attempting to quantify intra- or intermolecular interactions within the cell membranes that exhibit very different mechanical properties (Fig. 4). Spherical AFM tips are common for tissue or bulk soma measurements. Smaller cone or pyramidal AFM tips are typically used for measurements on particular points across the cell or for mapping the elastic modulus over entire regions [18]. However, for the examination of neuron elasticity and accurate determinations of Young's modulus values, a very hard probe should be used in such measurements. The elastic modulus of material (or Young's modulus) is defined as the ratio between the uniaxial forces applied to the sample and the uniaxial deformation that it undergoes, i.e. it describes how sample responds to force.

By raster-scanning the tip across the surface and recording the change in force as a function of position, a map of surface topography and other properties can be generated.

The AFM is useful for obtaining 3D topographic information of samples with lateral resolution (in the x/y plane) down to 0.3 nm and vertical resolution (in the z-axis) down to 0.1 nm [26]. These samples include clusters of atoms and molecules [27], individual macromolecules [28], and biological species (cells, DNA, proteins) [29, 30]. Sample preparation for AFM imaging is minimal. AFM can operate in gas, ambient, and fluid environments; it can measure physical properties including elasticity, adhesion, hardness, molecular bond strength, surface friction and chemical functionality [18, 31-33].

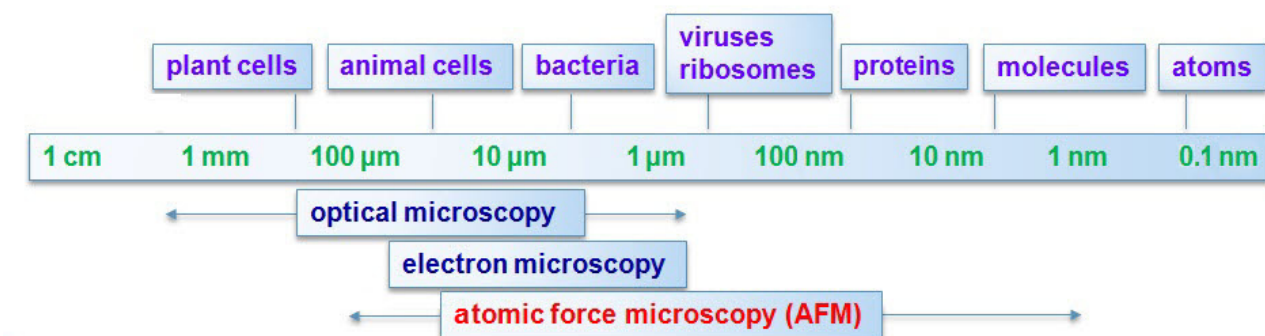


Figure 1. Size area comparison of AFM with other microscopies.

AFM modes of operation

The range of available operational modes is a unique characteristic of AFM in comparison with other microscopic techniques. The diversity of operational modes enables high-resolution imaging under physiologically relevant conditions, and offers the ability to directly measure protein-protein interactions, determine locations and distributions of certain binding sites, and measure stiffness [34]. The primary modes of AFM imaging are contact mode, tapping mode, and non-contact mode.

Contact mode is the most common mode of atomic force microscope operation and is useful for obtaining 3D topography of nanostructured surfaces. The tip and sample remain in close contact during the scanning process, and static deflection of the cantilever is measured. "Contact" represents the repulsive regime within the intermolecular force curve (Fig. 2). Most cantilevers have spring constants $< 1 \text{ N/m}$, which is less than the effective spring constant holding atoms together.

Tapping mode is the second mode of AFM operation in which the tip makes intermittent contact with the surface. During the tip scanning over the surface, the cantilever oscillates near its resonant frequency (from 7 Hz for contact mode to 525 Hz for tapping mode) by using a piezoelectric drive located inside its tip head. Tapping mode is usually preferred for the soft sample imaging (polymers, thin films) or for the structures that are weakly bound to

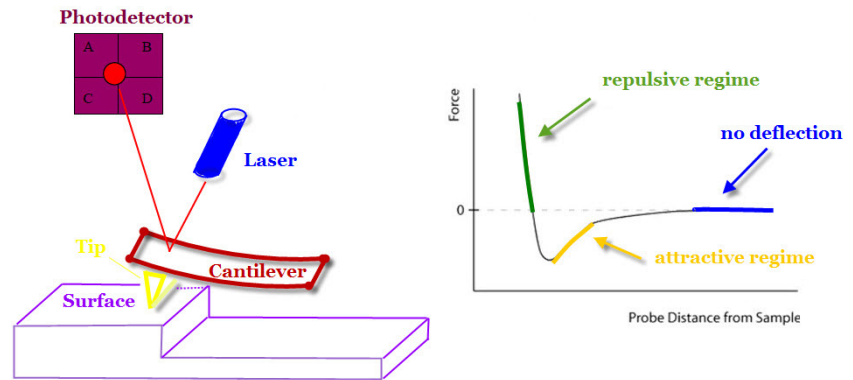


Figure 2. Schematics of an atomic force microscope and the force-distance curve characteristic of the interaction between the tip and sample.

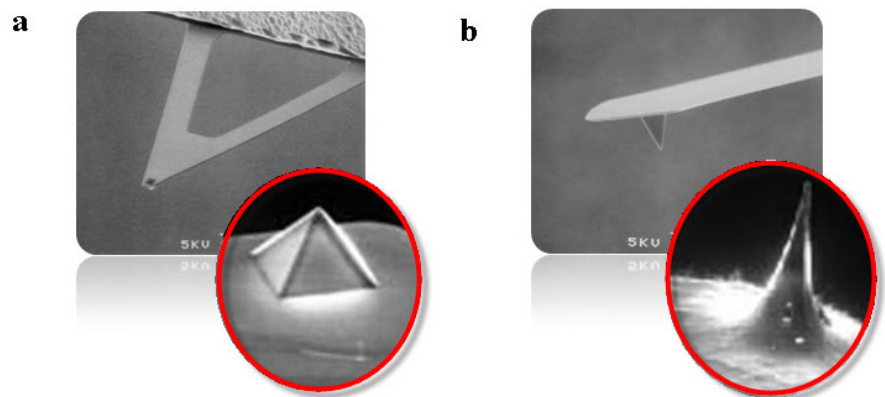


Figure 3. Scanning electron microscopy of the AFM probe. A) V-shaped silicon nitride probe for contact mode. B) Silicon-nitride probe for tapping mode imaging. Detailed description of the modes is given in the text.

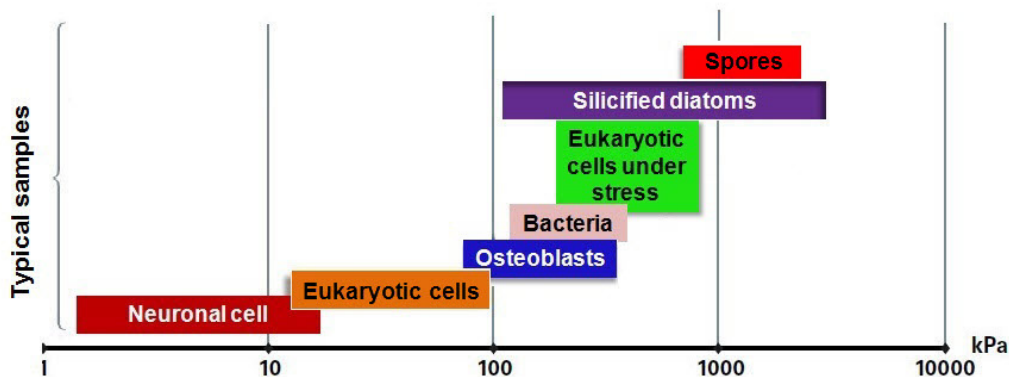


Figure 4. Range of Young's modulus values of various biological samples. Young's modulus is an indicator of a cell's response to stress (force). Depending on their type, eukaryotic cells can exhibit very different mechanical properties. Neurons are extremely soft (down to 1 kPa), whereas bone cells are as robust as bacteria.

the surface. The use of AFM on soft biological samples was significantly increased after the development of tapping mode [35]. Within the tapping mode, there are also two other types of image contrast mechanisms:

- *Amplitude imaging.* The feedback loop adjusts the z-piezo to retain the amplitude of the cantilever oscillation constant. The voltages needed to keep the amplitude constant can be compiled into an (error signal) image, and this imaging often provides high contrast between features on the surface.
- *Phase imaging.* The phase difference between the driven oscillations of the cantilever and the measured oscillations can be attributed to different material properties and provides information about qualitative differences in chemical composition, adhesion, and friction properties.

Non-contact mode also uses changes in the oscillation of the cantilever to produce the image. Cantilever oscillates above the sample surface at a distance within the attractive regime of the intermolecular force curve (Fig. 2). However, it uses changes in the attractive van der Waals forces that pull on the tip during approach to the surface to determine surface topography.

The choice of AFM mode is based on the surface characteristics of interest and on the sample hardness. Contact mode is most useful for hard surfaces, but there is a possibility for the contamination of the tip from the rest of the material on the surface or solution above the surface. Tapping mode is well suited for soft biological sample imaging and for samples with poor surface adhesion such as DNA. Non-contact mode is another mode useful for imaging of soft samples.

Topography and morphology of neurons

As mentioned, AFM provides a tool for imaging super soft biological samples, particularly with the emergence of tapping mode and force spectroscopy. AFM has been primarily used as a technique for quantitative imaging of surface

topography, such as topography of live or fixed cells, with molecular or supramolecular resolution [31, 36-38]. AFM cannot provide topographic information about structures that lie beneath the surface. As an advantage in the comparison with scanning electron microscopy, preparation of fixed cells for AFM is minimal, while living cells can be analysed in almost physiological conditions [39]. Cells can be kept at adequate temperatures, in nutrient buffers, or even at a recommended CO₂ concentration [40]. It is demonstrated that AFM probing does not affect neuronal health on a heated stage, although it provides time limits for experiments performed on non-heated stages [35].

Due to its very high spatial resolution, AFM reveals important structural information not accessible by other microscopic techniques. In neuroscience, such previously unreported architectures include transitory spines, ridges, and extensions of the soma and growth cone. In addition, minute hair-like extensions of the membrane along the walls of neuritic processes that shift in shape and density have been observed [36, 41]. The prominent vertical resolution of AFM is particularly important for obtaining images of these continuously appearing and disappearing cytoplasmic projections on neuronal somas and growth cones [36]. Dynamic arrangement of microfilaments and microtubules shapes these extensions on the order of minutes, during the period of time required for the AFM image collection. Thus, with AFM it is possible to monitor rapid changes in structure of live neurons over time.

Limitations of this technique include the necessity of neuronal adhesion to a 2D substrate, and occurrence of morphology changes during scanning. These constraints are particularly important when imaging active growth cones [18]. The neuronal growth cone is a highly motile structure at the distal part of an elongating axon. It receives multiple extracellular guidance cues via surface receptors, and translates that information into directional movements within the developing tissue. In addition to the development, it is of major importance to stimulate growth cone behaviour and promote axonal regeneration after neuronal injury. In contrast to neuronal

soma and neuron arborization, growth cones are subcellular structures particularly suitable for AFM analysis because they are flat and very strongly adhere to the substrate, at least in conditions obtainable with AFM [42]. AFM can provide a precise measurement on the nanometer scale of the shape of growth cones and its constituents, lamellipodia and filopodia. Furthermore, as AFM can physically interact with the cell under investigation, the AFM tip can be used to produce nanoscale injury (nano/micropuncture) to the plasmalemma of growth cones and cell somata. AFM can afterward be employed to monitor neuronal morphological response to this damage. McNally and Borgens found that injured somata excreted their degraded cytoplasm, which was evident as an enlarging pool formed beneath and around the cell [36]. Conversely, similar punctures to the membrane on the terminal end of the neurite, including the growth cone, did not lead to cell death, indicating the robust nature of axons in central nervous system.

By taking advantage to image neuronal cells over time, Laishram and coworkers analysed the morphology of growth cones of differentiating neurons from rat dorsal root ganglia (DRG) by combining conventional laser scanning confocal microscopy (LSCM) and AFM [37]. They superimposed images of immunofluorescent DRG growth cones labelled with actin and tubulin to high-resolution images of the external surface obtained with AFM. Analysis of obtained images revealed topographical structures with nanoscale dimensions, termed "invaginations" or "holes". Comparative analysis with LSCM images showed that these holes represent regions with slight actin- and tubulin-staining. These results show how the combination of LSCM and AFM may reveal structural details within a nanoscale dimension of DRG growth cones, which is difficult to resolve with conventional microscopy.

Neuronal biomechanics

AFM is also used for the characterization of the mechanical properties of neuronal cells, and has proven to be a highly versatile testing tool in mechanobiology, enabling the measurement of material properties at the cellular and

subcellular level over a large range of forces (from pN to nN levels), speeds (from quasistatic to dynamic levels), and length scales (from nm to μm) by using tips with different geometries [43].

Biomechanical properties are usually reported as cell elasticity. Cellular elasticity is ability to recover cellular shape following deformation due to external pressure. In general, the relationship between the force and deformation (stress and strain) is linear. Due to the non-invasive nature of elasticity measurements together with the ability to carry out measurements under near-physiological conditions, determination of cell elasticity has become interesting to many research areas. Determining the elastic modulus of a cell via AFM usually involves taking force vs. indentation curves on the sample, and is represented by Young's modulus. AFM has been used to determine nanomechanical properties of both live and fixed neurons. Fixed neurons yield Young's modulus values from 10 to hundreds of kPa, depending on the type of neuron under study, and the region of the cell that is measured [18]. As fixation increases the elastic modulus of the sample, measurements performed on live cells are of greater biological significance, although this restricts the time available for the measurements and maximum forces that can be applied. In addition, cell attachment to the substrate can be the problem, as cells and neuritic processes that are not well attached to surface may float around the cantilever [18, 21]. Furthermore, AFM elasticity measurements of cellular regions that are thicker than 300 nm are affected by the underlying substrate [35].

In living cells, elasticity is mostly determined by the components of the cytoskeleton, which is predominantly composed of actin filaments, neurofilaments and microtubules, and organelles. Areas of high elastic modulus measured in the neuronal cell bodies are areas of high microtubule density [44]. The cytoskeleton of a cell is constantly adapting based on the interactions with the local microenvironment, and this can be considered as an indicator of cell health [45]. In one study, topography and elastic modulus of DRG neurons have been studied before and after induced nerve injury,

showing higher elastic modulus in healthy growth cones (16–33 kPa) than in injured growth cones during regeneration [33]. The increase of the growth cone's membrane elasticity suggests a modification in the ratio of the main structural proteins that make the inner framework. Such studies are of great importance, as understanding the cellular and molecular mechanisms leading to improved neurite regrowth is a major step to propose a new therapy for nerve repair after injury (Martin et al., 2013). In another study, Ricci and coworkers used AFM to probe growth cones of a chick embryo's spinal cord neurons under vital conditions [42]. They quantitatively estimated the thickness of growth cone regions. In general, it is impossible to distinguish between a change in structure and a variation in stiffness without knowledge of the surface properties. By using force vs. indentation curves, it is feasible to distinguish these effects and estimate the thickness of the undeformed surface. By comparing their images with images generated by other techniques and described in the literature, they identified a central region and a peripheral region that are characterized by a different thickness and a different structural organization. Furthermore, they observed dot-like structures and identified them as clusters of adhesion molecules.

Axons are constantly exposed to mechanical stimulation by external and internal forces. When applied forces exceed a certain threshold, the result is irreversible injury and axonal degeneration. Magdesian *et al.* combined microfluidics with AFM and *in vivo* imaging to investigate the resistance of single axons to damage [40]. They developed a model using AFM to apply gradual forces to locally compress axons of rat hippocampal or DRG neurons grown in microfluidic chambers. They estimated the threshold forces required to 1) uncouple axonal transport without impairing axonal survival, and 2) compromise axonal survival in both individual and bundled axons. Although in both types of neurons similar morphological changes were observed under compression, their responses differed in time and intensity. Hippocampal axons completely recovered axonal transport with no detectable axonal loss when compressed with

pressures up to 65 ± 30 Pa for 10 min, while DRG axons can resist to pressures up to 540 ± 220 Pa. It turns out that the composition of the axonal cytoskeleton is critical to determine the axonal elasticity, and consequently, the degree of axonal susceptibility to damage. DRG axons contain approximately seven times more neurofilament than hippocampal axons, whereas actin and tubulin amounts are similar in both axonal types. These differences in cytoskeleton composition are likely reflected in the axonal viscoelastic properties and resistance to injury. To determine whether the differential susceptibility of hippocampal and DRG axons to mechanical injury is indeed influenced by differences in elasticity due to different cytoskeletal architecture, they further evaluated the elastic modulus of live axons using AFM and found that the elastic modulus of DRG axons was $\sim 20\%$ lower than that of hippocampal axons at every indentation depth.

AFM is also useful for studying mechanical properties of the cell during cell adhesion and for the quantification of adhesion forces. It provides an ideal technique for the characterization of mechanical contacts between cells, down to the level of single molecule [46]. Organization of cytoskeleton affects cell adhesion because intracellular cytoskeletal components are connected to the surface by adhesion receptors called integrins. Attachment to the surface is important for survival and growth of the cells as it determines cell shape, cytoskeletal organization, motility, differentiation and proliferation. AFM is often used to correlate elastic behaviour and cell migration or division. These studies are predominantly based on tapping mode, single-force curves, or force-volume measurements, as will be explained afterwards. The modulation of the cell properties consistent with the cytoskeletal organization differs as a function of the adhesion status of the cell. In particular, stronger adhesion corresponds to cells adherent to a surface coated with extracellular matrix proteins, while a weak adhesion was observed on a plastic surface [47]. Thus, the comparison of neuronal elasticity may serve as an indicator of cytoskeletal reorganization and the state of neuron adhesion.

The mechanical property measurements are also useful to study the physiology of sensory cells. Sensory hearing cells (outer hair cells) alter their length in response to changes in membrane potential. Due to this electromotility, outer hair cells probably expose the basilar membrane to force, resulting in cochlear amplification. In order to better understand the mechanism of amplification, AFM was used to analyse the mechanical properties of outer hair cells, as the force produced by their electromotility partly depends on the mechanical properties of the cell lateral wall [48].

Furthermore, it is demonstrated that AFM is a highly sensitive technique for detecting nanoscopic changes in the plasma membrane that result from oxidative damage, which is important hallmark of neurodegenerative diseases [49, 50]. In particular, AFM was used to characterize physical changes of average membrane roughness resulting from acute exposure to hyperoxia on human glioma cells [51]. Hyperoxic treatment caused a significant, graded increase in average membrane roughness relative to the control cells exposed to air. AFM scans also confirmed that normobaric hyperoxia induces plasma membrane blebbing that is similar in appearance to that observed after exogenously applied hydrogen peroxide, a strong oxidative stress-inducer. The size of most blebs (200 nm) indicates that the only way to observe them in living cells is by using AFM. Thus, AFM might be a powerful technique for a better understanding of the dynamic nature of membrane responses to oxidative stress in real time. In another study performed on neuronal SH-SY5Y cells, aimed to elucidate which oxidation state of ionic iron (ferrous (II) versus ferric (III)) is more toxic, AFM in tapping mode revealed membrane fenestration at higher concentrations and cell swelling at intermediate concentrations for the ferrous form of ionic iron [52]. Furthermore, AFM topography imaging on SH-SY5Y cells was used to analyse the effect on membrane integrity upon exposure to iron oxide magnetic nanoparticles, which have a wide range of biological applications. AFM topography images showed consistently that both the highly charged and the less charged magnetic

nanoparticles caused cell morphology changes, possibly due to membrane disruption and cytoskeleton remodelling. This report emphasises the potential of AFM in elucidating nanoparticles-cell membrane interactions and their application for *in vitro* characterisation of the biosafety of these particles [53].

AFM can also be used to monitor elasticity in changing environmental conditions and after exposure to neurotoxins [35]. In one study elastic modulus values were determined in the presence of a stabilizer of microtubules (paclitaxel/taxol), an inhibitor of microtubule polymerization (nocodazole), and inhibitor of myosin II contraction of the actin cytoskeleton by directly monitoring effects of these chemical modifiers on the stiffness of cytoskeletal components in live neuronal cells over time [44]. Thus, the nanomechanical properties of neuronal cells can be quantified by AFM and used to estimate the effect of drug treatment or different types of pathologies, as well as many natural processes, such as aging. Such studies could improve our understanding of neurodegenerative processes as changes of morphological and nanomechanical properties of neurons and growth cones, as well as cytoskeletal abnormalities, are core features of neurodegenerative diseases. Furthermore, a possibility to quantitatively investigate physical characteristics of the neuronal membrane, organelles and cytoskeleton may contribute to a better understanding of both neurodevelopment and neurotrauma.

Related to neurotrauma, novel testing protocols based on AFM were developed to enable the characterization of neural soma deformability over a range of indentation rates spanning three orders of magnitude – 10, 1, and 0.1 $\mu\text{m/s}$ [43]. These authors used modified spherical AFM probes to compress the neuronal cell bodies in load, unload, reload and relaxation conditions, and complemented rheological data with measurements of cell body morphology. The mechanical response measured for single cortical neurons showed marked nonlinearities in the strain and strain rate domains and substantial hysteresis. Based on experimental results and computational analysis, they proposed a model that quantifies the mechanical behaviour of cortical

neurons, i.e. estimates force and deformation magnitudes at the single neuron level after external mechanical inputs, which might be of importance in elucidating some of the key damage mechanisms involved in traumatic brain injury. In general, the model aimed to correlate empirical findings with measurable degrees of (hyper-) elastic resilience and viscosity at the cell level, allowing for large strain kinematics simulations of the cell behaviour.

In another study, a diagnostic approach was developed that provides clear feature definition in AFM images of neural cells on nanofibrillar tissue scaffolds [54]. Namely, cellular edges and processes are on the same order as the background nanofibers that present a feature definition problem. The diagnostic approach is based on analysis of discrete Fourier transforms of standard AFM section measurements. The combination of dynamic range enhancement with low-frequency component suppression was used to enhance feature definition. Clear feature definition of cells on scaffolds extends the usefulness of AFM imaging for use in regenerative medicine.

Combined AFM/ fluorescence technique

As previously mentioned, it is of great interest for neuronal studies to combine AFM and fluorescence labelling. For example, these methods enabled tracking of tubulin density in living, unstained neurons [44]. Furthermore, morphological responses of cerebral cortical astrocytes to nanofibrillar scaffolds, including filopodia, lamellipodia, stress fiber formation, and stellation were examined by AFM in combination with immunocytochemistry. The obtained results demonstrated that astrocytes cultured on the nanofibrillar scaffolds showed a unique response that included stellation, cell-cell interactions by stellate processes, and evidence of depression of RhoA, a GTPase regulator, indicating that the extracellular environment can trigger preferential activation of members of the Rho GTPase family with obvious morphological consequences [55]. Cell growth on nanofibrillar surfaces that approximate the extracellular

matrix environment is a research area in which the nanoscale resolution of AFM could give important biomedical insight. The data obtained by AFM might be useful to generate better growth substrates for application in regenerative medicine. The role of Rho GTPases has also been confirmed for adult neural stem cells that have important roles in learning and memory, but are negatively influenced by neurological diseases. Keung *et al.* used AFM to measure the elastic modulus of individual neural stem cells cultured on an extracellular matrix of defined stiffness [56]. They found that 12 hours after seeding, the cellular elastic modulus of neural stem cells varied strongly and monotonically with increasing stiffness of extracellular matrix, such that cells on the stiffest matrices exhibited an elastic modulus greater than those cultured on the most compliant extracellular matrices. Besides showing that stiffness of extracellular matrix modulates stiffness of neural stem cells, they demonstrated that extracellular matrix-derived mechanical signals act through Rho GTPases to activate the cellular contractility machinery in a key early window during differentiation to regulate neural stem cells lineage commitment. These authors also observed that culturing neural stem cells on increasingly stiff extracellular matrixes enhances RhoA and Cdc42 activation, and suppresses neurogenesis. Thus, with the aid of AFM, it is demonstrated that cellular mechanotransductive signalling pathways may sense and process extracellular mechanical information into intracellular mechanical responses. Furthermore, these results indicated Rho-GTPase-based mechanotransduction and cellular stiffness as biophysical regulators of neural stem cells fate *in vitro*.

In addition, AFM with the fluorescence imaging system has emerged as a highly sensitive method for the study of cell membrane receptor physiology. By using modified AFM probes, it is possible to analyse binding properties of specific receptors and channels along the neuronal surface. Namely, the ability to functionalise AFM probes with specific protein molecules enables the detection of individual ligand-receptor interactions. In particular, AFM has been used to detect alterations in the binding

properties (i.e. binding probability and force) of $\alpha 7$ -containing nicotinic acetylcholine receptors (nAChRs), and measure changes in the strength of protein-protein interaction, revealing functional changes during acute and prolonged exposure to nicotine. Internalization of Alexa Fluor 488-conjugated substance P was used to identify neurons expressing neurokinin-1 receptors (NK1R), a recognized marker of ventral respiratory group neurons [57]. In order to obtain functional changes in nAChRs, AFM probes were conjugated with anti- $\alpha 7$ subunit nAChR antibody, and used to cyclically interact with the soma surface of NK1R positive neurons. AFM force mode was used to measure binding forces between the plasma membrane and the AFM probe tip. Measurements were made of the frequency of antibody adhesion to the $\alpha 7$ -receptor subunit and of the detachment forces between the membrane-attached receptor and the AFM probe tip. Acute and prolonged nicotine exposure resulted in a significant reduction in the frequency of binding probability, which is presumably associated with a loss of nicotinic receptor function. In another interesting study, a specific anti-Met antibody (antibody to a tyrosine kinase receptor) was attached to the AFM tip and used to map Met in different cellular compartments of hippocampal pyramidal neurons. In concert with the role of hepatocyte growth factor (HGF) and its receptor Met in learning and synaptic plasticity, Met was found in brain regions which undergo extensive synaptic remodelling [58]. The results obtained with AFM indicated that multimeric activated Met was concentrated in the dendritic compartment, while the inactivated monomeric form of Met was prominent on the soma [58].

AFM force spectroscopy

AFM force spectroscopy is the basic AFM technique for the quantitative study of mechanical properties of cells and tissues [21]. By AFM force spectroscopy, the elastic properties of cells under near-physiological conditions can be measured. It is of great biomedical interest, because some cellular behaviours such as motility and aging can

manifest themselves as changes in the elasticity.

In AFM force spectroscopy, the tip-sample interaction forces are directly measured as a function of the gap between the tip and sample. The AFM tip is extended towards, and then retracted back from the surface. The deflection of the cantilever is monitored as a function of piezoelectric displacement. The results obtained are represented as a force-distance curve. Hence, the force vs. distance curve is a plot of the deflection of the cantilever versus the extension of the piezoelectric scanner that changes the distance between the sample and the tip. These measurements are used to obtain nanoscale contacts, atomic bonding, van der Waals forces, and single molecule stretching and rupture forces. Namely, when the tip is close to the membrane surface, in one moment the force is strong enough to rupture the membrane, which can be visualised as a jump in the force curve (Fig. 5). The depth of the jump corresponds to the thickness of the membrane and the force required to break the membrane is the force of membrane rupture. It represents the mechanical strength of the membrane. To obtain a statistically relevant histogram of the yield threshold forces, it is necessary to collect many force-distance curves that are measured in each point on the certain surface area and to use a consistent, robust procedure to identify and analyse those curves. The force needed to puncture the membrane is known as the yield threshold, and the membrane property that is actually measured by yield threshold may be called rigidity or hardness of the membrane.

Force spectroscopy measurements have been used to study ligand binding, antigen-antibody interactions and protein unfolding [59]. Force spectroscopy can also be used to analyze the adhesion of surface contaminants, as well as local variations in the elastic properties. However, force spectroscopy has some disadvantages such as low resolution and slower speed of acquisition.

AFM-based force spectroscopy can be combined with AFM imaging and fluorescence microscopy to produce systematic, high-resolution elasticity maps of live neurons. Three different types of live neuronal cells - cortical neurons obtained from rat embryos, embryonic

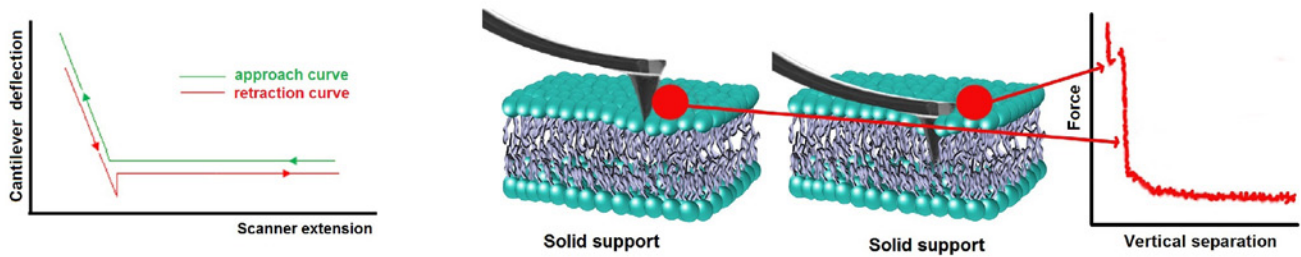


Figure 5. Simplified scheme of force vs. distance curve – the shapes, sizes, and distances are not to scale.

chick DRG neurons, and neurons derived from P19 mouse embryonic carcinoma stem cells, have been analysed using this approach [44]. By taking advantage of AFM to image and apply controllable forces to live neurons over time simultaneously, these authors monitored how the dynamics of axonal growth affects the stiffness maps of neuronal cell bodies. During the active neurite extension phase, an overall increase in the average values of the elastic modulus across the entire soma was observed, and these significant increases in stiffness were due to microtubule dynamics rather than changes in F-actin concentration, suggesting that microtubules have a major role in axonal extension. Furthermore, they showed that cortical and P19 neurons have similar elasticity maps, with elastic moduli in the range of 0.1–2 kPa, with typical average values of 0.4 kPa (P19 cells) and 0.2 kPa (cortical neurons). DRG neurons are stiffer than P19 and cortical cells, yielding elastic moduli in the range of 0.1–8 kPa, with typical average values of 0.9 kPa. Thus, AFM studies showed that due to the extreme complexity of both the mechanisms of neurite outgrowth and dynamics of cell cytoskeleton in response to mechanical properties, the different types of neurons respond distinctly to the same physical cues. Finally, Spedden *et al.* failed to find any variations in neuronal elastic properties in response to different local environment [44]. In particular, they reported no measurable influence of surface coating with poly-D-lysine, laminin and fibronectin on cell body elasticity for all three types of neurons. In fact, another study found that fixed neurons show dependence of the cell elastic modulus on the plating substrate [60]. These

authors used AFM with a robust force analysis approach to probe the mechanical properties of both neurites and the substrate at close proximity, and found that the lightly fixed primary spinal cord neurons exhibited different stiffness between the cell body and neurites. Furthermore, in comparison to the rigidity of the substrate, the stiffness of the neurites was lower, whereas that of the neuronal cell body was higher.

As already mentioned, the role of extracellular matrix molecules on cell adhesion, propagation, and differentiation is an important area of study. The majority of studies revealed that surfaces coated with different types of growth factors or extracellular matrix proteins can affect the dynamics of growth and adhesion of neuronal cells, and exert a profound effect on cell structure and function [61]. Physical stimuli, particularly substrate stiffness, may affect cellular behaviours in a cell-specific manner. Cells respond to the physical conditions of their microenvironment, and can adjust their own stiffness as a part of the adaptation to the substrate [60]. Primary neuronal behaviour is significantly different on soft and stiff substrates. Thus, Flanagan and coauthors examined the effect of substrate deformability on neuronal cell growth [62]. They prepared protein-laminated polyacrylamide gels with differing amounts of bisacrylamide to generate substrates of varying deformability with elastic moduli ranging from 500 to 5500 dyne/cm. Mouse spinal cord primary neuronal cells grown for several weeks on softer substrates formed more than three times as many branches as those grown on stiffer gels. Their results showed that mechanical properties of the substrate specifically direct the

formation of neurite branches, which are critical for appropriate synaptic connections during development and regeneration. Furthermore, laminin-coated soft gels encouraged attachment and growth of neurons while suppressing astrocyte growth. The stiffness of materials required for optimal neuronal growth, characterized by an elastic modulus of several hundred Pa, was in the range measured for the rat brain. Georges *et al.* dissected tissue from normal adult rat brain and measured stiffness by rheology [63]. Stiffness was expressed as a shear modulus and averaged ~330 Pa. Together, these data emphasize the importance of material substrate stiffness in the next generation of biomaterials intended to promote neuronal regeneration while simultaneously minimizing the ingrowth of astrocytes into the lesion area [63]. In another study, AFM force spectroscopy combined with bulk rheology has confirmed that in distinct cell compartments, such as soma and neurites, the mechanical properties differ (mostly because of the unequal distribution of organelles), and revealed that glial cells in central nervous system are softer than neuronal cells. This suggests that, at least in certain brain areas, glial cells act as a soft, compliant embedding for neurons, protecting them against mechanical trauma, and as a soft substrate required for neurite growth [64].

Single cell force spectroscopy: an advanced tool for neuronal studies

Various types of force spectroscopy exist. Some examples are single-molecule force spectroscopy (SMFS), and single-cell force spectroscopy (SCFM). As reviewed by Dufr ne *et al.*, single-molecule force spectroscopy

technique has been used to study how inter- and intramolecular interactions control the assembly and functional state of biomolecular machinery *in vitro* [34].

In a standard AFM-based single cell force spectroscopy (SCFS) experiment, the cantilever is moved to the cell with a constant speed while measuring the interaction force that increases upon contact with the cell membrane. The shape of the indentation curve provides information about the mechanical properties of the cell, mainly in terms of Young's modulus and elasticity. After a short delay (typically 1–5 s), the cantilever is moved away and a characteristic pattern is recorded from which several adhesion-related quantitative parameters can be extracted. Even the application of SCFS in cellular biology is only at its beginning, it is a promising technique to bridge the gap between physiological state and molecular determinants [65].

By using SCFS, the rigidity of cultured N2a and HT22 neuronal cells has been measured as a function of amyloid β_{1-42} ($A\beta_{1-42}$) protein treatment [66]. This study showed that $A\beta_{1-42}$ oligomers led to significant cellular stiffening, while disaggregated or fibrillar forms of $A\beta_{1-42}$ showed much less change. For example, a 90–360% higher force was required to reach 80% deformation for N2a cells, so these authors concluded that incorporation of oligomer into cellular membrane probably resulted in an increase in the Young's modulus of the membrane. Hence, such measurements provide deeper, quantitative insight into interactions between cells and $A\beta_{1-42}$ oligomers, which might be of relevance for understanding pathological cascade of events in Alzheimer's disease.

The application of SCFS to neuroblastoma studies suggested a correlation between the malignancy stage and the cell microadhesion, thus allowing investigation of the molecular basis of such correlation [65]. Mescola *et al.* also applied this technique to study organisation of the cytoskeleton of neuroblastoma cells, and found a correlation between cytoskeleton organisation and malignant potential that can be exploited towards a new method for *in vitro* quantification of malignancy of neuroblastoma cells [67].

Furthermore, SCFS AFM has been efficiently applied to demonstrate a conformational polymorphism of characteristic protein monomers in different neurodegenerative disorders and to identify that mechanostable conformers correlate with severity of cellular dysfunction and represent an interesting early diagnostic marker and therapeutic target [68].

Force volume

As explained in A Practical Guide to Scanning Probe Microscopy (Veeco Instruments Inc., 2005), force volume imaging is a technique for studying correlations between tip-sample forces and surface features by collecting a data set containing both topographic data and force-distance curves. As the topographic data is collected, an array of force-distance curves is also collected. This type of data is then called force volume. Each force curve is measured at a unique x,y position in the scan area. The force volume data set can be seen as a stack of horizontal slices, each slice representing the

array of force data at a given height. A single force volume image represents one of these slices, showing the x,y distribution of the force data over the scan area at that height (Fig. 6). Comparing the topography image to force volume images at various heights can reveal information about the lateral distribution of different surface and material properties, including magnetic, electrostatic, and chemical properties.

The potential of the force volume data has been revealed by studying changes in intracellular free calcium, one of the earliest cellular responses subsequent to mechanical stimulation of individual isolated porcine DRG neurons [69]. The change in intracellular free calcium concentration is located upstream of further biochemical cascades and triggers downstream signalling. Primary calcium signals induced by local deformation of the plasma membrane from a soma were rapidly followed by secondary calcium responses in neighbouring cells, demonstrating that the cultured DRG neurons are functionally

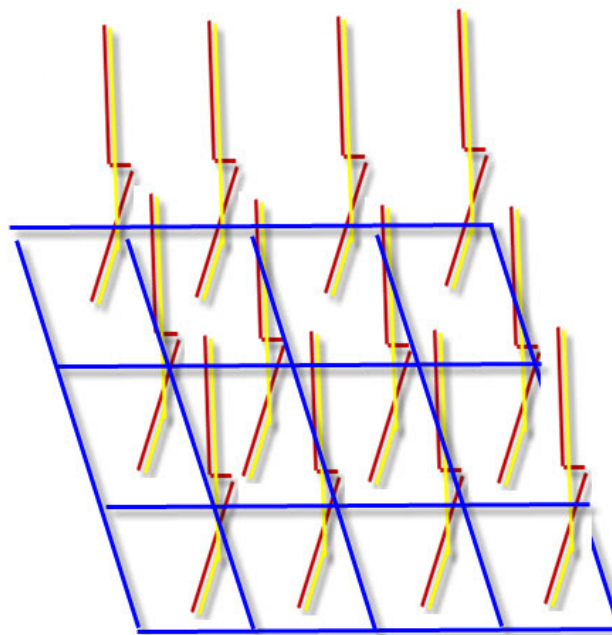


Figure 6. Schematic representation of AFM force volume map obtained by measuring the force – distance curve at any point in a two-dimensional array. Force volume technique describes how molecules and cells respond to mechanical stimuli or exert forces. The data obtained on force along two-dimensional array is compared to the image topography at various heights to obtain the information about the lateral distribution of different surface properties.

connected [69]. When a mechanical force has been applied to one of the two peripheral endings of the same DRG neuron, the stimulus elicited a secondary calcium response even in neurons located far from the soma, but in close vicinity to the non-indented neurite. Hence, in this study authors demonstrated the ability of the AFM to measure biological responses with a high spatial and temporal resolution during precise mechanical indentation. Finally, a new cell shape index (CSI) analysis system has been developed using force volume AFM height images of cells cultured on different substrates. The new CSI revealed quantitative cell spreading data not included in the conventional CSI. The quantitative morphology measurement of untreated and dibutyl-cyclic adenosine monophosphate (dBcAMP)-treated cerebral cortical astrocytes was performed using the new and conventional CSI, and the results showed that quantitative astrocyte spreading and stellation behaviour has been induced by variations in nanophysical properties [70].

Possibilities of new PeakForce Quantitative Nanomechanics

The new PeakForce Quantitative Nanomechanics (QNM) is by far the most powerful and quantitative high-resolution AFM technique to probe quantitative chemical and mechanical properties of living biological samples with an acquisition speed comparable to tapping mode. The number of different mechanical properties that can be characterized exceeds those of other commonly used AFM modes. It has great potential for new applications in biology and medicine, particularly in the field of neuronal cell research and neurodegenerative diseases. PeakForce QNM enables direct extraction of quantitative nanomechanical data from biological samples without sample damage. In PeakForce QNM tapping, the probe is oscillating at a low frequency, while force curves are generating each time the AFM tip taps on the sample's surface. While the probe is oscillating, a force curve is recorded for each pixel of the image. Many quantitative parameters

can be extracted in real time from each of these force curves. These make PeakForce QNM the most powerful AFM technique for material property characterization at the nanoscale that is available today. As already emphasized, it is also suitable for neuronal studies and its possibilities are shown in Fig. 7, which presents the PeakForce QNM measurements of untreated P19 neurons, performed by Bruker Laboratories (Karlsruhe, Germany). The new AFM mode reveals not only topographic 2D- (A) and 3D-height image (B), but also 2D- (C) and 3D-Young's modulus (D), the Young's modulus distribution within the cell (E), as well as the deformation image (F) and adhesion image (G). The cell displays regions of high elastic modulus localized at the lower end (bright areas in the figure). The topographic image provides fine structural details within distinct domains of various heights and/or various densities. The Young's modulus map describes the two-dimensional area response to applied forces. For example, the increase of the growth cone

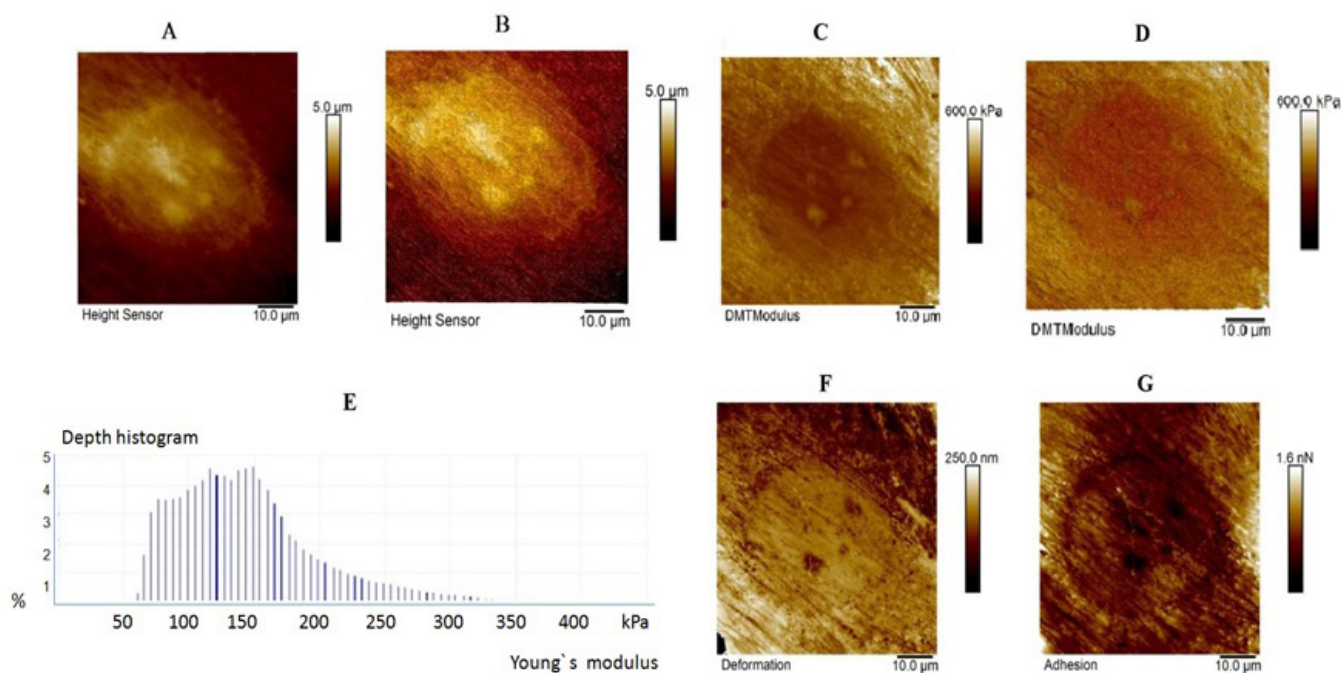


Figure 7. A) 2D and B) 3D topography of neuron soma obtained during elastic modulus mapping. C) 2D-high resolution elastic modulus map of cell shown in A. D) 3D-high resolution elastic modulus map of cell shown in A. E) Young's modulus distribution within the cell. F) Deformation and G) adhesion maps. Atomic Force Microscopy imaging was carried out on a BioScope Resolve (Bruker, Karlsruhe, Germany)) operated in PeakForce Tapping mode. AFM probe used was ScanAsyst-Fluid (Bruker) and imaging was done in PBS buffer.

membrane elasticity suggests a modification of the inner protein networks [33]. The elastic deformation image enables identification of the damage on selected neuron domains as a function of the loading force applied by the tip. Analysis of the deformation image facilitates the distinction of a locally damaged cell from a healthy cell, either on the cell membrane or its cytoskeleton [33]. Finally, integrin-mediated adhesion reveals sensing processes in neurons. Sensing of the nanomechanical properties of the external environments may determine cell behaviours, such as differentiation and branching. It has been experimentally determined that increasing substrate stiffness (~2-80 kPa) can result in sequestered neurite branching and increased branch length [71]. Adhesion of the modified tips to the surface during force curves can be recorded with higher values of adhesion indicating higher concentrations of active substances. Such measurements might be employed to track signalling activity in neurons [18]. Glutamate-mediated neuronal degeneration has also been examined by AFM imaging and surface roughness analysis using the newest PeakForce QNM AFM [72]. AFM was employed to measure the 3D structure and mechanical properties of live SH-SY5Y cells after stimulation of *N*-methyl-D-aspartate (NMDA) receptors. A significant increase in surface roughness and stiffness of the cell was observed after NMDA treatment. This study further advanced the understanding of the neurodegenerative process to elucidate the pathways and mechanisms that govern the neurodegeneration, which might facilitate development of novel therapeutic strategies.

Future directions

The development of AFM represents a major breakthrough in the study of individual biological samples, such as nucleic acids, proteins, and protein complexes. More recently the atomic force microscope has been used to investigate eukaryotic cells. The AFM has proven to be a very effective tool for studying topographic and mechanical features of living and fixed neuronal cells. The high-resolution capability of the AFM to obtain physiologically

relevant elastic modulus data on living cells may have important applications in the future. For example, continued exploration of changes in structure and elastic modulus of diseased and dysfunctional neurons may help scientists gain additional insight into how different neuronal defects contribute to various neurological diseases. As recent studies have shown that certain types of neurons exhibit preferential growth on substrates close to their own average elastic modulus, expanded data on live neuron elastic modulus under varying conditions might be useful to generate better growth substrates for different types of neurons, with applications in regenerative medicine [18].

The use of conventional AFM for imaging dynamics of live biological samples has been challenging, as it takes several minutes to acquire an image and a tip motion can damage the sample. To optimize AFM for the investigation of dynamical biological systems, each component of AFM can be modified to improve the speed of AFM scanning. As a result, high-speed AFM (HS-AFM) achieved scanning speeds several orders of magnitude faster than that of conventional AFM, thus enabling monitoring of conformational dynamics of single proteins on substrates with a subsecond temporal resolution. However, application of this technique to imaging nanostructure of live mammalian cells is hindered by the fact that the length scale of mammalian cells is orders of magnitude larger than that of proteins. Shibata *et al.* further improved HS-AFM by fabricating an extremely long tip on the cantilever [73]. This modification enabled them to image nanometer-scale morphogenesis in hippocampal neurons for more than tens of minutes without damage to cells. By applying this technique, they observed rapid filopodia extension and retraction on thin dendrites. Pits undergoing cycles between open and closed states were also observed on the dendritic surface, probably indicating spontaneous endocytosis events. Thus, the successful observations of nanostructural dynamics in live neurons may open the possibility to visualize the morphology of synapse plasticity at nanometer resolution in real time in the near future.

Conclusions

AFM enables the highest resolution neuronal imaging and the most complete analysis of nanomechanical properties of living neuronal cells with minimal sample damage. It provides detailed insight into physical structure and biochemical interactions in both physiological and pathophysiological conditions. The unique advantage of AFM is in the opportunity to perform simultaneous measurement of the nanomechanical properties of neurons and visualisation of important cellular structures, such as cytoskeleton. To enable better insight in cellular mechanisms related to cytoskeleton network, AFM analysis can be combined with confocal fluorescence microscopy or application of a drug that alters cytoskeleton components.

In the recent years, researchers have recognized the potential of AFM as an advanced tool in neuroscience, particularly for investigations of nanostructural and biomechanical properties of neurons. The major advantage of AFM is its ability to perform measurements without preparation, and in an almost natural environment, which maximally preserves morphological and functional properties of neuronal surface. AFM offers great possibilities in many research areas, and reveals structural detail not obtainable by other microscopies. AFM can be used to produce nanoscale injury and monitor growth cone activity during neuronal repair, improving our understanding of cellular and molecular mechanisms of regrowth and injury. AFM is also valuable in observing cell motility and cytoskeletal rearrangement. AFM may help in elucidating the role of extracellular matrix molecules on growth and adhesion, with potential applications in regenerative medicine. By monitoring physical interactions of the oligomers with the plasma membrane and nanomechanical membrane response to oxidative damage, AFM highly improved our understanding of neurodegenerative processes that eventually will open new avenues in the development of effective therapeutic strategies. AFM also facilitated imaging of surface distribution of various receptors across living neuronal cells, as well as their physiological responses to different

drugs and neurotoxins. The functionalised AFM probes enable detection of individual ligand-receptor interaction and study of cell membrane receptor physiology. The field of AFM applications in neuronal studies is continuously developing, and new advanced scanning techniques will certainly open up new applications in the future.

Without any doubts, AFM brings tremendous opportunities and deep insight into the various physiological and pathophysiological processes. However, at the very end of this

review, it should be emphasized that AFM technique has also considerable limitations. Data obtained by AFM are just as good as the model under study. Living neurons studied by AFM are isolated from the effects of other tissues and organ structures, and deprived of interactions with other brain cells. Hence, their topographical and biomechanical properties may not be identical to cells in the intact system. Nevertheless, data obtained by AFM are valuable starting point for predictions of *in vivo* processes and indispensable in many

research areas, particularly in the fields of neuronal injury and repair, development, and neurodegeneration.

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